CYSTEINE PROTEASE AND INHIBITORS FOR PREVENTION AND TREATMENT OF NEUROCYSTICEROCOSIS

This application claims the benefit of U.S. Provisional Application Serial No. 60/130,338, filed April 21, 1999, which is incorporated herein by reference in its entirety.

Statement of Government Rights

This invention was made with government support under a grant from the US/Mexico Foundation, Grant No. 42-H-94, and the N.I.H. Molecular Parasitology Training Grant For Cell Biology of Parasites and Vectors, Grant No. AIO7322. The U.S. Government may have certain rights in this invention.

Background

Neurocysticercosis is one of the most common parasitic disease of the human central nervous system and the leading cause of epileptiform seizures in many parts of the Third World. Presently, no completely efficacious treatments nor vaccines for neurocystercercosis exist (Garcia et al., J. Infec. Dis. 175:486-489 (1997)). Caused by cysts of the pork tapeworm, *Taenia solium,* the disease is estimated to affect 50 million people worldwide, although some epidemiological studies suggest that this figure may be underestimated. It is the most likely reason that epilepsy is twice as common in developing as opposed to developed countries of the world. Some symptoms associated with neurocysticercosis include seizures, headaches, visual problems, confusion, and psychosis. However, there is a prepatant asymptomatic period which can last for 10 years or more, and usually approximates the life expectancy of the tissue cysts. Evidence suggests that tissue cysts suppress host inflammatory responses to complete their life cycles, whereas clinical symptoms emerge during the

interval between cyst degeneration and death (Evans et al., Emerg. Infect. Dis. 3:403-405 (1997)).

With the increase in immigration, cysticercosis is an emerging infectious disease in the United States. The number of neurocystercercosis cases in the U.S. is estimated to be greater than 1000 per year , and this number appears to be on the rise. For example, 10% of neurology admissions at Los Angeles County USC Medical Center in the past three years were for neurocysticercosis, which amounted to about 120 cases/year. Surely, the underlying rate of infection is higher. Locally acquired cases have also been documented in Denver, San Diego, New York City, Chicago, and other unexpected locations. For example, 1.3% of an orthodox New York Jewish community were found to test positive for neurocystercercosis. Although orthodox Jews would not be expected to be at risk for infection because they shun pork, they were reported to have been infected through contamination from their immigrant Latino domestic helpers who were *Taeniasic* (tapeworm carriers). Four million people worldwide are reportedly infected with *Taeniasis*, harboring the adult tapeworm stage of *T. solium*. For every one of these victims, it is estimated that 10 or more people are infected with the cyst stage, i.e., are cysticercotic. In areas of high endemicity, almost anyone is at risk for neurocystercercosis, since the eggs can pervade the environment.

Humans are the only definitive host for the adult pork tapeworm, which remains in the intestine attached with its scolex. Humans acquire neurocystercercosis when they become accidental *intermediate* hosts to the cyst form of the parasite, commonly acquiring eggs through human fecal contamination or by direct contact with a tapeworm carrier. Pigs are the normal intermediate hosts. In humans, the cysts tend to localize more in the central nervous system, hence the prevalence of neurocystercercosis.

The International Task Force for Disease Eradication has identified neurocystercercosis as a target for eradication efforts since *T. solium* has only two hosts: humans and pigs. Although good porcine husbandry has met with

some success in interrupting transmission of *T. solium* in western Europe and the U.S., these approaches are doomed to failure in developing countries since poor farmers often prefer to sell infected pigs in an informal setting, rather than turning them over for possible condemnation. Some people are reported to actually prefer cysticercotic ("measly") pork to eat.

To date, no vaccines have been developed for any human parasites, including *T. solium*. Recent research has indicated that mass chemotherapy approaches are more promising and practical for the purposes of neurocystercercosis eradication. For example, an initial mass treatment with Praziquantel(TM) in Ecuador caused porcine cysticercosis to fall from 11.4 to 2.6%. Present treatment for active parenchymal neurocystercercosis commonly employs the antiparasitic compounds Praziquantel, Albendazole(TM), or a combination of both. A slight effect toward cyst resolution has been noted in patients who were treated with Praziquantel followed by subsequent treatment with Albendazole. However, some patients presented with worsening inflammatory reactions around the cysts with symptoms including nausea, vomiting, and headache; some patients developed cerebral edema. While some reports have shown that these drugs have demonstrated control of seizures in neurocysticercosis patients, it is not clear if these findings were a result of therapy or a result of patient selection bias. Consequently, the risks of developing acute inflammation, cerebral edema, and possibly chronic hydrocephalus associated with these agents is of significant concern. Although some studies suggest that Albendazole may be more effective than Praziquantel, it has also been linked to hematopoietic and hepatic dysfunction and may be teratogenic/mutagenic. Carpio and colleagues conducted the largest single randomized and blinded trial treatment of neurocystercercosis with Albendazole and Praziquantel, reported to date, and conclude that any benefits appear to be related to the natural course of the infection rather than any specific effect of drug treatment (Arch. Int. Med., 155: 1982-1988 (1995)). There appear to be no antiparasitic drugs that have been developed exclusively for treatment of

neurocystercercosis. Clearly, there is a need for a more efficacious and specific anti-neurocystercercosis drug, possibly to be optimized to work as an adjunct to existing therapies.

Summary of the Invention

A cyst wall cysteine proteinase has been discovered in the parasite *T. solium* that appears to play a significant biological role in the organism. The invention is thus directed to the *T. solium* cyst wall cysteine proteinase and a polynucleotide having a nucleotide sequence that encodes the cysteine proteinase (and its complement). The invention is further directed to a vaccine that includes either or both of a cyst wall cysteine proteinase (or an immunogenic polypeptide subunit thereof) or a polynucleotide having a nucleotide sequence encoding a cyst wall cysteine proteinase (or an immunogenic polypeptide subunit thereof). The cyst wall cysteine proteinase used in the vaccine is preferably derived from *T. solium* or *T. crassiceps*. Also included in the invention are methods of preparing and using the vaccine.

Inhibitors of the activity of cyst wall cysteine proteinase have also been discovered. The invention is thus also directed to a pharmaceutical composition that includes an inhibitor molecule that inhibits the activity of a cyst wall cysteine protease, together with a pharmaceutically acceptable carrier. The inhibitor molecule is preferably a peptide or peptidomimetic compound, and preferably inhibits cyst wall cysteine proteinase derived from *T. solium* or *T. crassiceps*. In a preferred embodiment, the inhibitor peptide or peptidomimetic compound includes $(Xaa)_n$-Yaa-Zaa-R; wherein Xaa and Zaa are each independently any amino acid; Yaa is a hydrophobic amino acid; R comprises a nucleophilic moiety; and n = 0-5. More preferably, the inhibitor peptide or peptidomimetic compound contains a blocking group at the N-terminus. The pharmaceutical composition is useful for treating human neurocysticercosis or porcine cysticercosis. Methods of preparing and using the inhibitory compound

and the pharmaceutical composition are also included in the invention. For example, the invention includes a method for inhibiting cyst wall cysteine proteinase activity that involves contacting a cyst wall cysteine proteinase with an inhibitor molecule. The method of inhibiting cyst wall cysteine proteinase can be performed in cell free environment, in cell culture, in an organ, in a tissue, or in a whole animal, such as a human or a pig.

Also included in the invention is a method for identifying an inhibitor of *Taenia* cysteine proteinase activity. A candidate inhibitor is combined with a *Taenia* cysteine proteinase to form a mixture. The proteinase substrate Z-Phe-Arg-7-amino-4-trifluoromethlycoumarin is then added to the mixture, and the extent to which the proteinase substrate is cleaved is determined. It should be understood that the components of the mixture can be added simultaneously or in any desired order. A reduction the extent of substrate cleavage compared to the extent of substrate cleavage in the absence of the candidate inhibitor is indicative of inhibition of *Taenia* cysteine proteinase activity.

The invention also includes a computer-assisted method for identifying an inhibitor of *Taenia* cysteine proteinase activity. In one embodiment, a computer model of the structure of an inhibitor of *Taenia* cysteine proteinase activity is supplied, then a structural library computationally or visually screened for candidate compounds having a structure similar to that of the inhibitor. Alternatively, a candidate compound having a structure similar to that of the inhibitor can be designed. Optionally, the candidate compound is for the ability to inhibit *Taenia* cysteine proteinase activity. In another embodiment of the computer-assisted method of drug design, the X-ray crystal structure of a *Taenia* cysteine proteinase is solved to yield a computer model of the *Taenia* cysteine proteinase, and a model of an inhibitor molecule is computationally or visually docked to the proteinase crystal structure at the binding site of the inhibitor. Intermolecular interactions between the inhibitor and the proteinase are then computationally or visually identified, and either a structural library is computationally or visually screened for candidate compounds having a structure

similar to that of the inhibitor or a candidate compound is computationally designed. Then, the intermolecular interactions between the candidate compound and the proteinase are computationally or visually evaluated, and the candidate compound is optionally assayed for the ability to inhibit *Taenia* cysteine proteinase activity.

Brief Description of the Figures

Figure 1 shows (a) 30 amino acid N-terminal sequence of *T. crassiceps* cysteine protease (SEQ ID NO:1); and (b) alignment of catalytic cysteine residue (*) with other cysteine proteases.

Figure 2 is a comparison of FPLC anion-exchange chromatography of active fractions from ACA 54 gel-filtration separations of a) *T. crassiceps* (Example I) and b) *T. solium* (Example II).

Figure 3 shows substrate specificity of the purified a) *T. crassiceps* (Example I) and b) *T. solium* (Example II) cysteine proteases assessed through cleavage of a panel of peptide substrates with different substitutions at P1-P3.

Figure 4 is Western blot showing degradation of IgG heavy chain by cysteine proteases of a) *T. crassiceps* (Example I) and b) *T. solium* (Example II).

Figure 5 shows stimulation of splenocytes removed from *T. crassiceps* infected mice by the purified *T. solium* enzyme.

Figure 6 shows inhibition of *T. solium* cysteine protease's cleavage of its primary substrate, Z-Phe-Arg-AFC, by various inhibitors.

Figure 7 shows scanning electron microscopy (SEM) of surface of cysts removed from mice treated with *Taenia* cysteine protease inhibitor and control mice. Magnifications are identical for both sides in each panel. Panel A (4000x): SEM shows a vigorous host immune response on cysts treated with Z-LLY-FMK. No immune cells were seen on cysts removed from untreated mice. Panel B (25,000x): SEM results show fibroblasts, holes in the tegument, and sloughed microtriches in cysts from treated mice. Microtriches in untreated cysts can be seen to be visibly longer. Visible anchors are were also noted for the

untreated cyst group whereas these anchors were not observed in microtriches from treated cysts.

Figure 8 shows *in vitro* cytotoxicity analysis of Z-LLL-FMK and Z-LLY-FMK inhibitors.

Detailed Description of the Invention

A cysteine proteinase that is capable of degrading host proteins, including human IgG, has been discovered in the cyst wall of the human parasite *T. solium*. *Taenia* cyst wall cysteine proteinases are significant to the life cycle of the parasite, thereby presenting important chemotherapeutic targets. Cyst wall cysteine proteinases of *T. solium* and *T. crassiceps* (the mouse parasite) are biochemically similar, and inhibitor molecules have been discovered that specifically inhibit the activity of both these enzymes. Inhibitors of *T. solium* and *T. crassiceps* cysteine proteinase are useful to treat or prevent infection of animal hosts, including humans.

Accordingly, the present invention is directed to a proteinase, preferably an isolated cyst wall cysteine proteinase, derived from *Taenia solium*, which proteinase is capable of cleaving the substrate Z-Phe-Arg-7-amino-4-trifluoromethylcoumarin (Z-Phe-Arg-AFC; where Z is the blocking group CH_5-CH_2-O-(C=O)- which is also known as "CBZ"). Z-Phe-Arg-AFC is commercially available from Enzyme Systems Products, Livermore CA, 94550. A protein, such as a proteinase, that is "derived from" *T. solium*, as that term is used herein, is to be broadly understood to include not only a protein that is physically isolated from *T. solium*, but also a protein having the same activity and, optionally, the same amino acid sequence as a protein thus isolated which is produced or obtained in any other manner, such as by means of chemical synthesis or recombinant DNA technology. Likewise, the term "*T. solium* protein" is to be understood to include a protein isolated from *T. solium*, together with synthetic or recombinant functional equivalents thereof. The terms

"proteinase" and "protease" are used interchangeably herein, and mean an enzyme capable of cleaving a protein at a bond, typically a peptide bond, that joins together two amino acids.

Preferably, the proteinase of the invention has one or more of the following characteristics:

(1) it is capable of rapidly and completely degrading the heavy and light chains of human IgG (for example, a nonlimiting amount of cysteine proteinase can completely degrade 1 ug of human IgG within about 120 minutes, preferably within about 45 minutes, as detected using a Western blot);

(2) it has a molecular weight of about 43 kD to about 65 kD;

(3) optimal proteinase activity occurs at acidic pH, about pH 4.8;

(4) it is thiol-activated; that is, it requires exogenous thiol groups, such as L-cysteine, for activity;

(5) the ratio Kcat/Km using Z-Phe-Arg-AFC as a substrate is essentially at the diffusion control limit (preferably greater than about 10^8 $M^{-1}s^{-1}$

(6) it has only one substrate binding site;

(7) it obeys Michaelis-Menten kinetics; that is, it is noncooperative;

(8) it is inhibited by cysteine proteinase inhibitors, but is not inhibited to the same extent by inhibitors of other types of proteases; and

The proteolytic activity of the newly identified proteinase of the human parasite, *T. solium*, appears to be essentially undistinguishable from the demonstrated activity of the analogous protein of the mouse parasite, *T. crassiceps* (White et al., J. Mol. Biochem. Parasitol., 85:243-253 (1997).

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The invention is further directed to an isolated nucleic acid molecule comprising a nucleic acid sequence that encodes a proteinase of the invention. The complement of such a nucleic acid molecule is also encompassed by the invention.

An "isolated" biomolecule, such as a polypeptide or polynucleotide, is a biomolecule that has been either removed from its natural environment, produced using recombinant techniques, or chemically or enzymatically

synthesized. Preferably, a biomolecule of this invention is purified, i.e., essentially free from any other biomolecules and associated cellular products or other impurities. The terms "polypeptide" or "polypeptide subunit," as used herein, refer to a polymer of amino acids and do not connote a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide, whether produced using recombinant techniques, chemical or enzymatic synthesis, or naturally occurring. This term also includes polypeptides that have been modified or derivatized, such as by glycosylation, acetylation, phosphorylation, and the like.

The present invention is further directed to an inhibitor of a cyst wall cysteine proteinase. The inhibitor molecule inhibits the activity of a cyst wall cysteine proteinase, preferably a *Taenia* cyst wall cysteine protease, more preferably a *T. solium* or *T. crassiceps* cyst wall cysteine proteinase. An inhibitor molecule is preferably a peptide (i.e., a molecule that contains two or amino acids or derivatized amino acids linked by a peptide bond) or a peptidometic compound, although it can contain one amino acid or derivatized amino acid. A "peptidomimetic" compound is a compound that functionally and/or structurally mimics a peptide, but that lacks one or more of the peptide bonds that characterize the peptide. Peptidomimetic compounds therefore not typically do not serve as substrate for proteases and are likely to be active *in vivo* for a longer period of time as compared to the analogous peptides. Unless otherwise specified, the term "peptide" when used herein in the context of an inhibitor peptide or inhibitor molecule includes peptides and peptidomimetic compounds.

In one preferred embodiment, the inhibitor molecule contains, covalently linked to its C-terminus, a nucleophilic chemical moiety. The nature of the nucleophile is not limiting and can include a carboxylic acid derivative, an amide derivative, a benzene ring derivative, a phenyl derivative, a pyridyl derivative, and the like. Suitable nucleophilic moieties include but are not limited to a

chloromethylketone (CMK), a fluoromethylketone (FMK), an alphaketo acid (AK), a ketoamide (KA), a ketoester (KE), a vinylsulfone (VS), a pyridyl group (Pyr), or any combination thereof. A preferred nucleophilic moiety is a fluoromethylketone (FMK). Preferably the nucleophilic moiety is also hydrophobic, thereby assisting the inhibitor molecule in crossing the blood-brain barrier. Additionally or alternatively, a preferred inhibitor peptide contains, at the P2 position (P2 being the second amino acid position, counted from the C-terminus), a hydrophobic amino acid, preferably an aliphatic hydrophobic amino acid, more preferably a leucine. In a preferred embodiment, a tyrosine or leucine is at the P1 position of the peptide; and/or leucine is at the P3 position, although it should be understood that any amino acids are well tolerated at the P1 and P3 positions. Particularly preferred inhibitor peptides include Xaa_n-Leu-Tyr-FMK and Xaa_n-Leu-Leu-FMK where n = 0-5; more preferably, n = 0-2, for example Z-Leu-Leu-Tyr-Fluoromethylketone (Z-LLY-FMK) and Z-Leu-Leu-Leu-Fluoromethylketone (Z-LLL-FMK), commercially available from Enzyme System Products, Livermore, CA. Other preferred inhibitor peptides include $(Ph)_2CHCO$-Leu-Phe-CO-$NHCH_2$-2-Pyridyl (Ph2-LF-KP); Z-Leu-Phe-CONH-Ethyl (Z-LF-KE); Z-Leu-Phe-CONH-4-Morphophinyl (Z-LF-KM); Z-Leu-Phe-CH_2-CH(OH)-C_6H_4-(4-OPh) (Z-LF-BPh); Boc-Leu-Phe-Vinylsulfone-Phenyl (B-LF-VS); and Z-Leu-Leu-Leu-Vinylsulfone-Phenyl (Z-LLL-VS).

The N-terminus of an inhibitor peptide or peptidomimetic of the invention is preferably derivatized. For example, it can be covalently linked to a blocking group, such as "Z" (CH_5-CH_2-O-(C=O)- which is also known as "CBZ") or "Boc" (t-butoxy). N-terminal blocking groups are well known in the art of synthetic organic chemistry and peptide synthesis.

The invention further provides methods for treating or preventing infection of an animal by *Taenia*. Preferably, the animal is a cow, a dog, a pig or a human. More preferably, the animal is a pig or a human and the method is effective for treating and preventing a *T. solium* infection. In one aspect, a pharmaceutical composition containing an inhibitor molecule of the invention is

administered therapeutically to an animal harboring a *Taenia* infection. In one embodiment of the therapeutic method, administration of the inhibitor molecule is effective to eliminate the parasite from the animal; in another embodiment, administration of the inhibitor molecule is effective to prevent or delay the appearance of cysticercosis or neurocysticercosis in the animal. In another aspect, a pharmaceutical composition containing an inhibitor molecule of the invention is administered prophylactically to an animal in advance of infection by the parasite. In one embodiment of the prophylactic method, administration of the inhibitor molecule is effective to prevent subsequent infection of the animal by the parasite. In another embodiment of the prophylactic method, administration of the inhibitor molecule is effective to prevent or delay the development of cysticercosis or neurocysticercosis in the animal after subsequent infection by the parasite. In yet another embodiment of the prophylactic method, administration of the inhibitor molecule is effective to prevent the death of the animal after subsequent infection by the parasite.

Both veterinary and human applications are contemplated, as the inhibitor molecule is expected to inhibit the activity of cyst wall cysteine protease derived from various species of *Taenia*, including *T. solium* and *T. crassiceps*. Veterinary applications include prophylactic and therapeutic treatment of domestic animals, which may also control the spread of infection into human populations. Preferably, the method of the invention comprises administration to a pig or human that is infected with *Taenia* or at risk of *Taenia* infection. In humans, the inhibitor is preferably introduced into the bloodstream, for example by intravenous injection or by ingestion. In pigs, the inhibitor is preferably administered in feed as a prophylactic or therapeutic agent against porcine cysticercosis, with the ultimate goal of interrupting the life cycle of *Taenia* at the porcine stage. Such a strategy could conceivably lead to the ultimate eradication of human neurocysticercosis.

Example V describes the use of inhibitor peptides of the invention to treat cysticercosis in the murine model of *Taenia* infection. These studies show

some of the highest reported protection levels in mice challenged with cysticerci, and the inhibitors were highly efficacious at low concentration levels. Moreover, mice which were treated with the inhibitor displayed no apparent toxicity effects upon visual examination. The results of these mouse studies strongly suggest that the inhibitor peptides are specific for a cyst wall cysteine protease which is present only in the parasite *Taenia*. In other words, the inhibitor peptide is not expected to significantly interfere with the activity of proteolytic enzymes in a mammalian host, such as a pig or a human. Accordingly, treatment of an infected animal with the inhibitor molecule of the invention is expected to be more specific than present treatments for neurocysticercosis, which include administration of Praziquantel and Albendazole. Both of these drugs were discovered by drug screening approaches and not by an approach directed toward inhibition of a particular *Taenia* enzyme activity, as in the present invention. Both drugs are not very effective and are accompanied by significant side effects in hosts.

The inhibitor molecules of the invention are readily formulated as pharmaceutical compositions for veterinary or human use. The pharmaceutical composition optionally includes excipients or diluents that are pharmaceutically acceptable as carriers and compatible with the inhibitor molecule. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the pharmaceutical composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, salts, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition. In a preferred embodiment, the pharmaceutical composition is formulated so as to allow or enhance transmission of the inhibitor molecule across the blood/brain barrier in humans. Methods of

making and using such pharmaceutical compositions are also included in the invention.

The inhibitor molecules can be formulated in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active compound into association with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as tablets, troches, capsules, lozenges, wafers, or cachets, each containing a predetermined amount of the inhibitor as a powder or granules, as liposomes containing the inhibitor, or as a solution or suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, an emulsion or a draught. Such compositions and preparations should contain at least about 0.1% active compound. The percentage of the compositions and preparations may be varied and may conveniently be between about 1% to about 60% of the weight of a given unit dosage form.

Tablets, troches, pills, capsules, and the like may also contain one or more of the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; an excipient such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, fructose, lactose or aspartame; and a natural or artificial flavoring agent. When the unit dosage form is a capsule, it may further contain a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac, or sugar and the like. A syrup or elixir may contain one or more of a sweetening agent, a preservative such as methyl- or propylparaben, an agent to retard crystallization

of the sugar, an agent to increase the solubility of any other ingredient, such as a polyhydric alcohol, for example glycerol or sorbitol, a dye, and flavoring agent. The material used in preparing any unit dosage form is substantially nontoxic in the amounts employed. The inhibitor may be incorporated into sustained-release preparations and devices.

The inhibitors of the invention can be incorporated directly into the food of the animal's diet, as an additive, supplement, or the like. Thus, the invention further provides a food product containing an inhibitor of the invention. Any food is suitable for this purpose, although processed foods already in use as sources of nutritional supplementation or fortification, such as animal feeds, breads, cereals, and the like, may be more convenient to use for this purpose.

Formulations suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the inhibitor, or dispersions of sterile powders comprising the inhibitor, which are preferably isotonic with the blood of the recipient. Isotonic agents that can be included in the liquid preparation include sugars, buffers, and sodium chloride. Solutions of the inhibitor can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions of the inhibitor can be prepared in water, ethanol, a polyol (such as glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, glycerol esters, and mixtures thereof. The ultimate dosage form is sterile, fluid and stable under the conditions of manufacture and storage. The necessary fluidity can be achieved, for example, by using liposomes, by employing the appropriate particle size in the case of dispersions, or by using surfactants. Sterilization of a liquid preparation can be achieved by any convenient method that preserves the activity of the inhibitor, preferably by filter sterilization. Preferred methods for preparing powders include vacuum drying and freeze drying of the sterile injectible solutions. Subsequent microbial contamination can be prevented using various antimicrobial agents, for example, antibacterial, antiviral and antifungal agents including parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. Delivery of the inhibitors over a prolonged period

can be achieved by including agents for delaying, for example, aluminum monostearate and gelatin.

Nasal spray formulations comprise purified aqueous solutions of the inhibitor with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes.

Formulations for rectal or vaginal administration may be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids.

Ophthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye.

Topical formulations comprise the inhibitor dissolved or suspended in one or more media such as mineral oil, petroleum, polyhydroxy alcohols or other bases used for topical pharmaceutical formulations. The compound of the invention is particularly suited to incorporation in a cosmetic lotion, crème, or sunscreen for use on the skin.

Useful dosages of the inhibitor molecule of the invention can be determined by comparing their *in vitro* activity and the *in vivo* activity in animals models. Methods for extrapolation of effective dosages of drugs in mice, and other animals, to humans are known in the art; for example, see U.S. Pat. No. 4,938,949, which is incorporated herein by reference, in its entirety.

It is expected that the inhibitor molecule of the invention will be superior to Praziquantel and Albendazole for treatment of neurocysticercosis as a result of its high efficacy and apparent specificity, which may lead to the effective use of lower drug dosages compared to those used for Praziquantel and Albendazole. In some applications, a treatment protocol may include administration of a combination of Praziquantel, Albendazole and/or an inhibitor molecule of the invention.

It should be understood that an inhibitor molecule of the invention may be effective to treat other parasitic infections, if the active site of the cyst wall cysteine proteinase is conserved among other metabolically important enzymes in other parasites.

The invention additionally provides a method for identifying an inhibitor of cyst wall cysteine proteinase activity. A candidate inhibitor is combined with a cyst wall cysteine proteinase derived from *Taenia*, preferably *T. solium* or *T. crassiceps*, to form a mixture. A detectable substrate for the proteinase, preferably the fluorogenic molecule Z-Phe-Arg-AFC, is then added to the mixture. After a period of time and under conditions sufficient to allow for cleavage, as described more fully hereinbelow, it is determined whether or not the substrate is cleaved. The absence of cleavage, i.e., recovery of intact substrate, identifies a promising inhibitor of cyst wall cysteine proteinase activity.

The three-dimensional conformations of the inhibitor peptides Z-LLY-FMK, Z-LLL-FMK, Ph2-LF-KP or any of the other inhibitor peptides described herein are amenable to computer-enhanced display and manipulation. These structures can be computationally constructed from readily accessible peptide libraries. It is within the scope of the invention to identify conformational analogs to Z-LLY-FMK or Z-LLL-FMK, for example, having different compositions, using computer-aided rational drug design techniques in common use in the field of computational biology. Computer-generated structural libraries of peptides and other small molecules are readily available, and can be computer-screened for conformational similarity to Z-LLY-FMK, or Z-LLL-FMK. Optionally, the X-ray crystal structure of a *Taenia* cysteine proteinase can be determined using methods well known in the art, and the coordinates thereof can be displayed on a computer. The active site can be identified, and potential inhibitors can be identified by putative binding to the active site via computer modeling techniques. Compounds thus identified as structurally similar to

Z-LLY-FMK or Z-LLL-FMK, or other compounds identified as potentially binding to the active site of the proteinase, are then subjected to *in vitro* testing according to the invention to determine whether they inhibit cyst wall cysteine proteinase activity as described below. The invention is understood to further include inhibitor molecules identified according the methods of the invention, in addition to the methods of identifying them.

The invention further provides a vaccine that is effective to treat or prevent infection of an animal by a cyst-forming parasite, such as *Taenia*. The vaccine can be a polypeptide vaccine or a polynucleotide vaccine, and can include one or more immunogenic components. The polynucleotide vaccine contains at least one polynucleotide having a nucleotide coding region that encodes a cyst wall cysteine proteinase or an immunogenic polypeptide subunit thereof. Analogously, a polypeptide vaccine contains at least one cyst wall cysteine proteinase or immunogenic polypeptide subunit thereof. An immunogenic polypeptide subunit of a cyst wall cysteine protease is one that elicits in an animal host an antibody-mediated immune response (i.e., a B cell response or humoral immunity), a cell-mediated immune response (i.e., a T cell response), or a combination thereof. The immunogenicity of a cysteine protease subunit can be evaluated, for example, using the assays set forth in the Examples. Optionally, the polypeptide or polynucleotide vaccine includes additional compounds that stimulate the host's immune system, as further described below. The invention should be understood as also including methods of making and using the polynucleotide and polypeptide vaccines.

The polynucleotide vaccine can contain DNA, RNA, a modified nucleic acid, or any combination thereof. Preferably, the polynucleotide vaccine comprises one or more cloning or expression vectors; more preferably, the vaccine comprises one or more expression vectors each capable of autonomous expression of a nucleotide coding region in a mammalian cell to produce at least one immunogenic polypeptide or cytokine, as further described below. An expression vector preferably includes a eukaryotic promoter sequence, more

preferably the nucleotide sequence of a strong eukaryotic promoter, operably linked to one or more coding regions. A promoter is a DNA fragment that acts as a regulatory signal and binds RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding sequence; transcription is the formation of an RNA chain in accordance with the genetic information contained in the DNA. A promoter is "operably linked" to a nucleic acid sequence if it is does, or can be used to, control or regulate transcription of that nucleic acid sequence. The invention is not limited by the use of any particular eukaryotic promoter, and a wide variety are known; preferably, however, the expression vector contains a CMV or RSV promoter. The promoter can be, but need not be, heterologous with respect to the host cell. The promoter used is preferably a constitutive promoter.

A vector useful in the polynucleotide vaccine of the present invention can be circular or linear, single-stranded or double stranded and can be a plasmid, cosmid, or episome but is preferably a plasmid. In a preferred embodiment, each nucleotide coding region (whether it encodes an immunogenic polypeptide or a cytokine) is on a separate vector; however, it is to be understood that one or more coding regions can be present on a single vector, and these coding regions can be under the control of a single or multiple promoters.

There are numerous plasmids known to those of ordinary skill in the art useful for the production of polynucleotide vaccines. Preferred embodiments of the polynucleotide vaccine of the invention employ constructs using the plasmids VR1012 (Vical Inc., San Diego CA), pCMVI.UBF3/2 (S. Johnston, University of Texas) or pcDNA3.1 (InVitrogen Corporation, Carlsbad, CA) as the vector. Plasmids VR1012 and pCMVI.UBF3/2 are particularly preferred. In addition, the vector construct can contain immunostimulatory sequences (ISS), such as unmethylated dCpG motifs, that stimulate the animal's immune system. Other possible additions to the polynucleotide vaccine constructs include nucleotide sequences encoding cytokines, such as granulocyte macrophage

colony stimulating factor (GM-CSF), interleukin-12 (IL-12) and co-stimulatory molecules such B7-1, B7-2, CD40. The cytokines can be used in various combinations to fine-tune the response of the animal's immune system, including both antibody and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to control or eliminate the *Taenia* infection. The polynucleotide vaccine can also encode a fusion product containing the immunogenic polypeptide and a molecule, such as CTLA-4, that directs the fusion product to antigen-presenting cells inside the host. Plasmid DNA can also be delivered using attenuated bacteria as delivery system, a method that is suitable for DNA vaccines that are administered orally. Bacteria are transformed with an independently replicating plasmid, which becomes released into the host cell cytoplasm following the death of the attenuated bacterium in the host cell. An alternative approach to delivering the polynucleotide to an animal involves the use of a viral or bacterial vector. Examples of suitable viral vectors include adenovirus, polio virus, pox viruses such as vaccinia, canary pox, and fowl pox, herpes viruses, including catfish herpes virus, adenovirus-associated vector, and retroviruses. Exemplary bacterial vectors include attenuated forms of *Salmonella*, *Shigella*, *Edwardsiella ictaluri*, *Yersinia ruckerii*, and *Listeria monocytogenes*.

The polynucleotide vaccine of the invention can be administered to the animal using any convenient method, such as intramuscular injection, topical or transdermal application to the animal's skin, or use of a gene gun, wherein particles coated with the polynucleotide vaccine are shot into the animal's skin. The amount of polynucleotide administered to the animal is affected by the nature, size and disease state of the animal as well as the delivery method; for example, typically less DNA is required for gene gun administration than for intramuscular injection. Further, if a polynucleotide encoding a cytokine is co-delivered with nucleotide coding regions encoding the immunogenic polypeptide, the amount of polynucleotide encoding the immunogenic polypeptide from in the vaccine is optionally reduced.

SUB B2

Hundreds of publications have now reported the efficacy of DNA vaccines in small and large animal models of infectious diseases, cancer and autoimmune diseases (J. Donnelly et al., Rev. Immunol. 15:617 (1997)). Vaccine dosages for humans can be readily extended from the murine models by one skilled in the art of genetic immunization, and a substantial literature on genetic immunization of humans is now available to the skilled practitioner. For example, Wang et al. (Science 282:476-480 (1998)) vaccinated humans with plasmid DNA encoding a malaria protein, and the same group has developed a plan for manufacturing and testing the efficacy of a multigene *Plasmodium falciparum* liver-stage DNA vaccine in humans (Hoffman et al., Immunol. Cell Biol. 75:376 (1997)). In general, the polynucleotide vaccine of the invention is administered in dosages that contain the smallest amount of polynucleotide necessary for effective immunization. It is typically administered to human subjects in dosages containing about 20 μg to about 2500 μg plasmid DNA; in some instances 500 μg or more of plasmid DNA may be indicated. Typically the vaccine is administered in two or more injections at time intervals, for example at four week intervals.

The polypeptide vaccine of the invention, in addition to containing cyst wall cysteine proteinase or an immunogenic polypeptide subunit thereof, can include an adjuvant in order to further stimulate the animal's immune system. Adjuvants suitable for human and veterinary use are well known in the medical arts. Like the polynucleotide vaccine, the polypeptide vaccine can be administered to the animal using any convenient method, such as intramuscular or intraperitoneal injection, topical administration, oral or intranasal administration, inhalation, perfusion and the like, as described above for the administration of the inhibitor molecule. The amount of polypeptide administered to the animal is affected by the nature, size and disease state of the animal, as well as by the delivery method. Useful dosages of the polypeptide vaccine for humans can be readily determined by evaluating its activity *in vivo* activity in mice.

Accordingly, the invention further provides a method for vaccinating an animal against infection by *Taenia*. Preferably, the animal is a cow, a dog, a pig or a human. More preferably, the animal is a pig or a human and the method is effective for treating and preventing a *T. solium* infection. In one aspect, the vaccine is administered therapeutically to an animal harboring a *Taenia* infection. In one embodiment of the therapeutic method, administration of the vaccine is effective to eliminate the parasite from the animal; in another embodiment, administration of the vaccine is effective to prevent or delay the appearance of cysticercosis or neurocysticercosis in the animal. In another aspect, the vaccine is administered prophylactically to an animal in advance of infection by the parasite. In one embodiment of the prophylactic method, administration of the vaccine is effective to prevent subsequent infection of the animal by the parasite. In another embodiment of the prophylactic method, administration of the vaccine is effective to prevent or delay the development of cysticercosis or neurocysticercosis in the animal after subsequent infection by the parasite. In yet another embodiment of the prophylactic method, administration of the vaccine is effective to prevent the death of the animal after subsequent infection by the parasite. Both veterinary and human applications are contemplated, as the vaccine is expected to be effective against various species of *Taenia*, including *T. solium* and *T. crassiceps*. Veterinary applications include prophylactic and therapeutic treatment of domestic animals, which may also control the spread of infection into human populations. Preferably, the method of the invention comprises administration of the vaccine to a pig or human that is infected with *Taenia* or at risk of *Taenia* infection. n pigs, the polynucleotide or polypeptide vaccine is preferably administered in feed.

The invention further contemplates administration to an animal of different vaccines and/or inhibitor molecules in a serial protocol. For example, a plasmid-based DNA vaccine as described herein can be administered to a animal to "prime" the immune system, followed by the one or more administrations of a

polypeptide vaccine, a viral vaccine (e.g., vaccinia vector carrying the genes that encode the immunogenic polypeptide and, optionally, cytokines) and/or a pharmaceutical composition comprising an inhibitor of cyst wall cysteine protease activity as identified according to the invention. The order of administration of the different types of compositions, and the nature of the compositions administered in any given dose (e.g., polypeptide vaccine, plasmid vaccine, viral vector vaccine, inhibitor molecule) can be readily determined by one of skill in the art to invoke the most effective immune response in the animal.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

Example I.

Purification and Characterization of a Cysteine Protease in the Murine Parasite *T. crassiceps*

A *T. crassiceps* cyst wall cysteine protease was purified to homogeneity (White et al., Mol. Biochem. Parasitol., 85:243-253 (1997)). The enzyme has an unusually high turnover ability for synthetic substrates and degrades human IgG rapidly *in vitro*. The molecule may play a key role in immune evasion by *Taenia*, a significant survival tactic employed by the parasite.

Because of its significant biological similarity to *T. solium*, rapid growth rates (> 100 cysts after 4 weeks of infection), and simple maintenance in BALB/c mice, *T. crassiceps* is commonly utilized as a model system for the study of the human parasite, *T. solium* (Hayunga et al., J. Parasitol.75: 638-642 (1989); Ambrosio et al., Arch. Med. Res. 25:325-330 (1994); White et al., Infect. Agents Dis. 1:185-193 (1992)). *T. crassiceps*' natural hosts include rodents

(intermediate hosts) and canines (definitive hosts). We purified a predominant *T. crassiceps* cysteine protease to homogeneity, as confirmed by its N-terminus sequence, and its presentation as a single band on silver stained gels of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme was solubilized from whole parasites with acid extraction, whereas little cysteine protease activity was found in excretory fluid, a finding also noted by others (Hayunga et al., J. Parasitol.75: 638-642 (1989); Ambrosio et al., Arch. Med. Res. 25:325-330 (1994)). Since acid extraction was able to dissociate most of the enzyme, in comparison to detergent solubilization, we hypothesized that the protease was located in the cyst wall, but was most likely not in the integral membrane. These observations were later supported by transmission electron microscopy, which localized cysteine protease activity to lysosomal vesicles and the cyst wall (Khalil et al., J. Parisitol. 84:513-515 (1998)). It is thus likely that this cysteine protease is located in the cyst wall of *Taenia*, rendering it accessible as a chemotherapeutical target.

Gel filtration (AcA 54 gel filtration column) and Fast Protein Liquid Chromatography (F.P.L.C.) anion exchange (Mono Q) chromatography were used to purify the cysteine protease 682-fold from the initial acid extract. The purified enzyme eluted at approximately 13% salt into 1 active sharp peak by anion exchange, and corresponded to 43 kDa based upon the expected molecular weight of the eluted fraction and the single band obtained by silver staining. (13% salt is significant since the similar *Taenia solium* cysteine protease also elutes at this percentage). Purity was further confirmed by an N-terminus sequence. Purity was further confirmed by an N-terminus sequence. Unambiguous results were obtained on the first 52 amino acids (Fig. 1(b)). An N-terminus sequence on material obtained from a second purification of *T. crassiceps* cysteine protease confirmed the sequence of the first 30 amino acids (Fig. 1a). Although the N-terminus sequence contains a cysteine at position 23, which is the catalytic cysteine in the papain superfamily, it does not contain the consensus pattern in enzymes of this superfamily (Hayunga et al., J. Parasitol.75:

638-642 (1989)). However, the *T. crassiceps* cysteine protease most closely resembles enzymes in the papain family, in comparison to sequences of other cysteine protease families (Fig. 1b). Thus, this enzyme may represent a new class of cysteine proteases with greatest homology to the papain superfamily.

The purified cysteine protease was assayed for proteolytic activity using a synthetic peptide substrate linked to the C-terminus of 7-amino-4-trifluoromethyl coumarin (Z-Phe-Arg-AFC), substantially as described in White et al. (Mol. Biochem. Parasitol., 85:243-253 (1997)). Purified protease (0.1 ug) is added to a saturating amount of Z-Phe-Arg-AFC (1ug/500uL assay buffer) in 0.4M citrate supplemented with 10mM cysteine, at pH 4.9. The mixture is incubate for 18 hours at 37C. The purified cysteine protease was characterized by unequivocal thiol dependence (a biochemical characteristic of all cysteine proteases), inhibition by cysteine protease inhibitors, a pI of 5.27, and a substrate specificity profile demonstrating pronounced cleavage of Z-Phe-Arg-AFC (see Example II). The purified protease was marked by an unusually high Kcat/Km (substrate turnover efficacy) on the synthetic peptide substrate, Z-Phe-Arg-AFC , equivalent to 2.2 X 10^8 $M^{-1}s^{-1}$, which is an order of magnitude that approximates the enzymatic "diffusion control limit" which some investigators have likened to "catalytically perfect" enzymes (Voet, Biochemistry, 2nd ed, John Wiley and Sons, eds. 14:371-400 (1995)). Very few enzymes have been reported with such a high catalytic Kcat/Km ratio. Most enzymes with these characteristics tend to be quick metabolic enzymes (e.g. hexokinase and triose phosphate isomerase), capable of instantly catalyzing reactions during every encounter with substrate. Thus, the impressive efficacy of the *Taenia* cysteine protease in turning over substrate with similar magnitudes was suggestive of an enzyme with a biological significance to the parasite (such as metabolism).

The activity of the *T. crassiceps* protease was inhibited by the irreversible cysteine protease inhibitor, E64 (L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane), indicating that the purified enzyme was a cysteine protease. The enzyme's Km was substantially increased, indicating

substantial loss of affinity of the cysteine protease for Z-Phe-Arg-AFC substrate. This result is consistent with a covalent interaction between E64 and the purified enzyme, as is typically characteristic for the interaction of E64 and cysteine proteases. In order to obtain these kinetic parameters, assays were set up such that Km and Vm were calculated respectively from the x and y intercepts of a double reciprocal Lineweaver-Burke plot of substrate concentration (moles) versus protease velocity (mol h^{-1}). Kcat was calculated from the inverse of Vmax.

Example II.

Purification and Characterization of a Cysteine Protease in the Human Parasite, *T. solium*

A cysteine protease from the human parasite, *T. solium*, which uses the pig as an intermediate host, was purified and characterized. This protease is similar, although not identical, to the cysteine protease derived in Example I from *T. crassiceps*, whose natural hosts are rodents, and which grows very well in the BALB/c mouse. The data, however, are compelling that the *active sites* of the two proteases are highly homologous (which is the most important premise for a specific inhibitor). A protease inhibitor specifically directed against the *T. solium* cysteine protease is thus expected to specifically inhibit the cysteine protease in *T. crassiceps* thereby permitting testing for inhibitor efficacy in the mouse model.

T. solium cysts were obtained from wild cysticercotic pigs occupying the village of Guerrero in Mexico. As in *T. crassiceps*, most of the cysteine protease activity of the *T. solium* cysts could be released by acid extraction, without the need for detergent solubilization. When detergent solubilization was employed, the specific activity in the acid extracts was still approximately 5-fold higher. Thus, like the *T. crassiceps* cysteine protease, the *T. solium* cysteine protease is likely located in the cyst wall, but is not an integral membrane protein.

The purification protocol used in *T. crassiceps* was used to purify the *T.*

solium cysteine protease. After acid extraction, gel filtration chromatography was employed and the active fraction eluted at 50-55 kDa, thereby suggesting that the *T. solium* cysteine protease is slightly larger than the *T. crassiceps* cysteine protease (43 kDa). Optical density readings on FPLC anion exchange, showed that the profiles of the *T. solium* and *T. crassiceps* enzymes are practically superimposable (Fig. 2). Active fractions from the AcA 54 gel filtration column were fractionated further by FPLC anion exchange chromatography using a Mono Q column. The two enzymes were eluted with identical gradients (dashed line) of 10 mM Tris, pH 7.6 (solution A) and 10 mM Tris, pH 7.6 plus 1 M NaCl (solution B). One ml fractions were collected and assayed with Z-Phe-Arg-AFC substrate (dotted line). Absorbance was monitored at 280 nm (solid line). As can be observed, both enzymes elute between 12-14% salt, suggesting that these enzymes have a highly similar and conserved charge conformation. The *T. solium* cysteine protease electromigrated as a single band on silver stained SDS-PAGE gels.

These data suggest that there are substantial similarities between the *T. crassiceps* and *T. solium* cysteine proteases. Further evidence suggests that the active sites are homologous. First, the pH optimum of the *T. solium* cysteine protease is identical to the pH optimum of the *T. crassiceps* cysteine protease (pH 4.9). Moreover, the substrate cleavage patterns are similar. For both enzymes, Z-Phe-Arg-AFC (Z-FR-AFC) is the most significantly cleaved substrate in comparison to a panel of synthetic substrates (Fig. 3). Next to Z-FR-AFC, it can be seen that Lys-Ala-AFC (KA-AFC) and Arg-AFC (R-AFC) are the next best cleavable substrates by the *T. solium* protease. These two substrates are also the next best cleavable substrates for the *T. crassiceps* enzyme. The overall activity with respect to the other substrates (e.g., LG-AFC, ZRR-AFC) is not high enough in either enzyme. This data showing similar cleavage patterns between the two enzymes further confirms the homologous nature of the two proteases' active sites.

Like the *T. crassiceps* cysteine protease active site, the *T. solium* cysteine protease is unequivocally thiol dependent, and is completely inhibited by the absence of cysteine. As shown in Table 1, both cysteine proteases were inhibited by cysteine protease inhibitors but not significantly (>50%) by inhibitors of other catalytic classes. For example, inhibitors of the epoxy class of cysteine protease inhibitors (ie: E64 and EP459 (L-trans-epoxysuccinyl-leucylamido(4-methyl)butane)) completely abolish activity. $HgCl_2$ inhibits both enzymes in a concentration dependent manner, as is characteristic of cysteine proteases. Near total inhibition is noted with the chloromethane inhibitors tosyl lysyl chloromethyl ketone (TLCK) and tosylphenylanyl chloromethyl ketone (TPCK), which are known to inhibit serine proteases and some cysteine proteases. No significant inhibition is noted by inhibitors of other classes including PMSF, 1,10 phenanthroline, ethylenediaminetetracetic acid (EDTA) or pepstatin. In the table, one representative experiment is shown, but a minimum of three experiments were performed. Inhibitors were incubated with enzyme for 30 minutes before the addition of substrate. Fluorometric readings were assessed after overnight incubation.

Table 1. Sensitivity of (a) *T. crassiceps* (Example I) and (b) *T. solium* cysteine proteases to inhibition by a panel of inhibitors when Z-Phe-Arg-AFC is used as the substrate

a)

Inhibitor	Catalytic class	Concentration	% Inhibition ± SD*
No cysteine			100
E-64	Cysteine	10^{-5} M	95 ± 3.0
		10^{-7} M	98 ± 1.5
		10^{-8} M	99 ± 1.0
Ep459	Cysteine	10^{-7} M	99 ± 1.1
Ep475	Cysteine	10^{-6} M	99 ± 0.9
Iodoacetate	Cysteine	10^{-3} M	99 ± 0.7
$HgCl_2$	Cysteine	10^{-4} M	3 ± 2.1
		10^{-3} M	40 ± 29
		4×10^{-3} M	77 ± 24
PMSF	Serine	2×10^{-3} M	47 ± 32
APMSF	Serine	10^{-4} M	0 ± 0
TLCK	Serine/cysteine	10^{-4} M	96 ± 3.1
TPCK	Serine/cysteine	10^{-4} M	96 ± 3.5
1,10 Phenanthroline	Metallo-	2×10^{-3} M	35 ± 38
EDTA	Metallo-	2×10^{-3} M	27 ± 38
Pepstatin	Aspartic	10^{-6} M	34 ± 34

b)

Inhibitor*	Catalytic class	Concentration	% Inhibition ± SD
No Cysteine			100
E-64	Cysteine	10^{-6} M	100 ± 2.7
		10^{-7} M	100 ± 0.2
		10^{-8} M	94 ± 0.9
Iodoacetate	Cysteine	10^{-3} M	100 ± 0.0
HgCl2	Cysteine	10^{-4} M	9 ± 3.1
		10^{-3} M	41 ± 10.0
		4×10^{-3} M	81 ± 1.4
PMSF	Serine	2×10^{-3} M	29 ± 10.0
TLCK	Serine/cysteine	10^{-5} M	93 ± 1.0
TPCK	Serine/cysteine	10^{-4} M	93 ± 1.0
1,10 Phenanthroline	Metallo	2×10^{-3} M	49 ± 10
EDTA	Metallo	1×10^{-2} M	13 ± 5.0
		2×10^{-3} M	8 ± 2.0
Pepstatin	Aspartic	1 X 10-5	34 ± 4.0
		1 X 10-7	39 ± 16
DCI			38 ± 11

Specific cysteine protease inhibitors (e.g., Suc-Leu-Tyr-AFC) abrogate the ability of the *T. solium* and *T. crassiceps* cysteine proteases to cleave human IgG on western blots (see Fig. 4b), when human IgG is used as a substrate. Thus, both enzyme active sites are marked by highly homologous inhibitor profiles.

In addition, the Kcat/Km ratio for the *T. solium* cysteine protease active site, on Z-Phe-Arg-AFC is equal to 2.84 X 10^9 M^{-1} s^{-1} and is close to the Kcat/Km ratio of the *T. crassiceps* cysteine protease (2.2 X 10^8 M^{-1} s^{-1}). The *T. solium* cysteine protease is thus also within the logarithmic range of the diffusion control limit of 10^8-10^9 for "catalytically perfect" enzymes. Consequently, the *T. solium* cysteine protease also demonstrates a remarkable ability to turn over substrate, and we thus believe this to be evidence that such an efficacious enzyme must be significant to this human parasite.

Finally, like the *T. crassiceps* enzyme, the *T. solium* enzyme is also characterized by the capability to degrade the heavy and light chains of human IgG (Fig. 4), and the ability to do it quickly. Human IgG was incubated for 18 hours with a) *T. crassiceps* acid extract or b) *T. solium* purified cysteine protease (CP). Incubated tubes were subsequently fractionated by SDS-PAGE under reducing conditions, blotted onto PVDF membrane, and visualized using biotinylated anti-IgG (heavy chain/light chain), biotin-peroxidase conjugated-strepavidin complex and enhanced chemiluminescence. Top and bottom arrows indicate heavy and light chains, respectively, in panel A. Only the heavy chain is shown in Panel B. The absence of heavy and light chains suggests that the proteins are broken down into peptides. In another experiment, we noted that like *T. crassiceps*, the *T. solium* enzyme accomplishes complete IgG degradation by 30 minutes. This degradation was thiol dependent, and inhibited by the cysteine protease inhibitor, E64, but not by inhibitors of other catalytic classes. When the specific inhibitor, Z-LLY-FMK (Example IV)) was incubated with the *T. solium* cysteine protease in the presence of IgG, degradation of the antibody was inhibited. The *T. solium* cysteine protease also appears to be biased for human IgG degradation. For example, it did not degrade human albumin when this substrate was employed at the concentration that was used in the human IgG degradation studies.

Collectively, our data suggest that the *T. solium* enzyme is biochemically and biologically nearly identical to the *T. crassiceps* enzyme, with regard to active site catalysis. Specific inhibitors that inhibit the *T. crassiceps* cysteine protease also inhibit the *T. solium* cysteine protease (Example IV). In summary, evidence that supports the similarity of the enzyme active sites includes: highly similar pH optima, inhibitor profiles, substrate profiles, identical elution patterns off of anion exchange column, similar degradation of IgG, and inhibition by highly specific inhibitors. Consequently, the *T. crassiceps* cyst can confidently be used as a model system to study biological effects of protease inhibitors against the *T. solium* cysteine protease.

Proposed interactions of Taenia cysteine proteases with the host's immune system

The cyst wall cysteine proteases of *Taenia* may play a significant role in *Taenia's* evasion of host immunity. *Taenia* cysts survive for many years in asymptomatic intermediate hosts. Thus, *Taenia* cysts must somehow evade host immune responses. Moreover, they must find host protein sources to degrade in order to obtain essential amino acid nutrients. We postulate a mechanism of how this may occur, beginning with immune evasion. However, it is to be understood that the present invention, insofar as it relates to methods and compounds for inhibiting the activity of a *Taenia* cysteine protease, is not intended to be limited by any particular mechanism or mode of action.

It has previously been suggested that IgG traffics through the blood brain barrier or is produced in the brain. An Fc receptor has been identified on the *Taenia* cyst wall, indicating that IgG uptake may be an active process (Kalinna et al., Parasitol.106:289-296 (1993)). We postulate that IgG is routed to the cyst lysosomes in the cyst wall internuncial processes and tegumentary cytons. Pathways demonstrating protein shuttling to the lysosomes followed by protein degradation are known in *T. crassiceps* (Ambrosio et al., Arch. Med. Res. 25:325-330 (1994)). The cyst wall is where the cysteine protease is also

localized. After transport to the lysosomes, we hypothesize that the IgG would be degraded in the lysosomes. Hayunga et al. (Hayunga et al., J. Parasitol. 75: 638-642 (1989)) have observed degraded IgG units in *T. crassiceps* lysosomal vesicles. IgG is subsequently broken down by the specialized cysteine protease into peptides, or possibly even amino acids in the lysosomes. Thus, the IgG degrading cysteine protease may aid the cyst by protecting it from destructive host IgGs (complement-fixing IgG2a is associated to dying metacestodes).

Eventually, the benefits of accessibility to IgG -derived peptide or amino acids "lying around" in the lysosome may have become a significant metabolic advantage. Indeed, the cysteine protease may help the parasite actually *exploit* the host immune response, not just disable it. Due to the impressive efficiency of the cysteine protease's ability to degrade IgG, antibodies may have become a good source of nutrients for the parasite *in vivo*, as has been suggested (White et al., Mol. Biochem. Parasitol., 85:243-253 (1997)). The general importance of the cysteine protease in growing parasites is illustrated by our transmission electron microscope (TEM) studies (Khalil et al., J. Parisitol. 84:513-515 (1998)) which show greater cysteine protease activity in smaller, metabolically active and growing cysticerci than in larger, older ones. The correlation between increased cyst growth and increased cysteine protease activity in these smaller cysts is consistent with the suggestion that the enzyme may play a key role in breaking down proteins, like IgG, for parasite nutrition.

In the end, *Taenia* cysts may have become sensitized to a balance where a certain level of IgG in the host serum was actually beneficial metabolically, but too much could be destructive, immunologically. Perhaps there is a baseline physiological IgG level which is beneficial (IgG uptake in *T. crassiceps* cysts is shown to be saturable at physiological serum levels (Siebert et al., Exp. Parasitol. 48:64-174 (1979))). Thus, *Taenia* cysts may have a need for a certain level of IgGs for immune exploitation, but are harmed by concentration beyond this. Perhaps, this is why the cysteine protease, which is highly antigenic (Example III), is not located on the cyst wall surface, but is rather *within* the cyst

wall. Consequently, the cysts may have evolved molecular mechanisms to control this balance. For example, a secretory *Taenia* glycoprotein (Villa et al., Parisotol. 112:561-570 (1996)) appears to modulate the shift from a host T helper 1 (Th1) cellular mediated immune response to a humoral T helper 2 response (Th2), favoring increased antibody production which may be used by the parasites for nutrition (this shift may also aid the parasites in avoiding a destructive Th1 cellular response, for which they have no defenses). Alternatively, diminished secretion of these molecules may slow down the shift from Th1 to Th2 allowing the parasites another means to control their immunological environment (and thus, physiological IgG levels).

SUB B3 cont

We thus undertook to design or identify (Example IV) specific inhibitors of *Taenia* cyst wall cysteine protease that potentially function using one or both aspects of a dual mode of action. First, specific inhibition of the cysteine protease may allow host immunoglobulins to recognize cysts thereby "unmasking" them to the immune system. In Example VI, we show how cysts from mice treated with specific inhibitors against the cysteine protease are coated by immune cells whereas cysts from nontreated mice are "clean." A second mode may be that inhibition of the cysteine protease may weaken the cyst by depriving it of IgG derived amino acids, thus impairing it metabolically. A "weakened" cyst would also more apt to be recognized by the immune system and possibly destroyed or at least prevented from further growth and/or proliferation. As shown in Example V our specific cysteine protease inhibitor treatments protected mice from cysticercosis, suggesting that cyst growth (by budding) was compromised.

Notably, the cysteine protease has been localized in these identical regions in which the degraded IgG was reported. Transmission electron microscopy (TEM) studies identified cysteine protease activity which cleaved the cysteine protease substrate, Z-Phe-Arg-methoxynaphthylamide (MNA) (Khalil et al., J. Parisitol. 84:513-515 (1998)) in acidic and lysosomal vacuoles of the cyst wall in *T. crassiceps*.. Free methoxynapthalymide was coupled to

osmium and p-rosanilin, and cleaved from Z-Phe-Arg at the substrate's carboxy side on the arginine residue. Compared to control without substrate, cysticerci incubated with substrate showed electron dense deposits in round vesicles, whereas most of these vesicles were found primarily within the cyst wall tegumentary cytons and internuncial processes. This study thus localized the cysteine protease to the cyst wall. When the cysticerci were incubated with the cysteine protease inhibitor, E64, prior to substrate addition, the electron dense bodies were fewer and less dense than in cysticerci without the inhibitor. Thus, the cysteine protease activity here was partially inhibited by E64, further supporting the presence of the cysteine protease in this region. It is hypothesized that inhibition was only partial with E64 because the concentration of this inhibitor within the acid vesicles may have been too low since the concentration of enzyme in the vesicles was higher than in our purification yields. Our important conclusions here are that 1) the cysteine protease appears localized to the same cyst wall regions where degraded IgGs have been observed, and that 2) a cysteine protease inhibitor was able to reduce the cysteine protease's activity *in situ*. This latter observation suggests the promising possibility that specifically designed protease inhibitors may be able to permeate and consequently, inhibit cysteine protease activity in the *Taenia* cyst wall.

Moreover, the pH of the lysosomes (pH 4.9) is the identical pH optimum for activity of the cysteine protease on the synthetic substrate, Z-Phe-Arg-AFC,and the activity of the cysteine protease is totally dependent upon exogenous reducing groups, which are also present in the lysosomes. Although Z-Phe-Arg-AFC is cleaved by cysteine proteases as well as by serine proteases like kallikreins, serine proteases do not optimally function at acidic pH and they are thiol dependent. Also, prior studies failed to identify serine protease activity in *Taenia* cysticerci walls with similar peptides. Thus, the proteolytic activity detected here with Z-Phe-Arg-AFC at acidic pH in the reducing environment of the lysosomes is most likely due to the cysteine protease that we purified from

the Taenia cyst wall. We also observed that the cysteine protease which we purified is the only one in the cyst wall.

The *Taenia* cysteine proteases cleave human IgG optimally at lysosomal pH and only in the presence of reducing groups, *in vitro*. It is thus notable that this enzyme was solely responsible for complete human IgG digestion on western blots, in comparison to other proteases present in the original acid extract of the purification protocol. The observation that both the heavy chain and light chains could be completely cleaved by the cysteine protease, suggests that the chains were reduced to peptides or amino acids, which are not visible on SDS-PAGE gels. Dose response experiments showed that the purified cysteine protease completely degraded both heavy and light chains of human IgG at a molar ratio up to 1:30 (enzyme: IgG), whereas partial degradation of IgG occurred at molar ratios up to 1:3000. Complete degradation was accomplished within only 45 minutes and was completely inhibited by the cysteine protease inhibitor, E64. As mentioned, E64 suppresses cysteine protease activity in the *Taenia* cyst wall *in situ*, suggesting that specifically designed cysteine protease inhibitors should also suppress IgG degradation by the cysteine protease. (Indeed, specific inhibition of the homologous *T. solium* cysteine protease is noted for Leu-Tyr-AFC-Fig 4b). Moreover, the substrate specificity profile of the purified enzyme most closely resembles cathepsin L-like cysteine proteases, which are mammalian lysosomal enzymes, and have also been linked to IgG degradation. These data are therefore compelling that the *T. crassiceps* cysteine protease efficiently degrades host IgG substrates.

Our data also suggest that the *Taenia* cysteine protease may also be preferentially selective for IgG degradation. For example, we have recently isolated a secretory cysteine protease from *T. crassiceps*, with a pH optimum of 6.3, which appears to cause only minor cleavage of the human IgG heavy chain but not the light chain. By comparison, the cyst wall cysteine protease appears to degrade both chains. Based upon these data, we believe that the *Taenia* cyst

wall cysteine protease significantly contributes to lysosomal degradation of host IgG in *Taenia*, and may also be specialized for this process.

In summary, the *Taenia* cyst wall cysteine protease's unusually high kinetic turnover ratio, its potentially key role in parasite nutrition and host immune evasion, its accessible location within the cyst wall (but not too deep within the cyst), and the fact that its activity can be reduced by cysteine protease inhibitors *in situ*, render this enzyme a promising target for antiparasitic chemotherapy.

Example III.

Vaccination of BALB/c Mice with the *T. solium* Cysteine Protease

BALB/c mice were vaccinated with the purified (eluted off the MonoQ anion exchange column) and partially purified (eluted off ACA 54 gel filtration column) *T. solium* cysteine protease (Example II) in Freund's Complete Adjuvant. Mice were subsequently boosted with the same preparations two weeks later. Five and a half months following the booster, the mice were challenged with *T. crassiceps* cysts followed by euthanization after 6 weeks of active infection. As can be seen in Table 2 below, mice vaccinated with the purified cysteine protease were protected 72% from cysticercosis, whereas mice vaccinated with the partially purified preparation, were protected 50%. The *T. solium* cysteine protease thus appears to be able to induce a cross-protective immune response in BALB/c mice, and this response correlates to the purity of the cysteine protease. This observation supports the characterization of these enzymes as antigenically similar.

Table 2. Protection of BALB/c mice from T. crassiceps cystercercosis infection, after immunization with the purified (ACA54 followed by Mono Q) and partially purified (ACA54 only) *T. solium* cysteine proteinase

	Cyst #	*AVG*	*STD*	*% Protection*	*Cyst Volume*	*AVG*	*STD*
MonoQ (pure)	43	38.6	18.6	**71.8**	1.6	1.3	.6
	59				1.8		
	14				.5		
ACA54 (partially pure)	62	69	5.3	49.8	2	2.1	.12
	75				2.3		
	70				2.1		
Positive	158	137.3	18.6		7	7.2	.21
(no vaccine)	113				7.5		
	141				7.1		

We also obtained splenocytes from *T. crassiceps* infected mice and stimulated them with the purified *T. solium* cysteine protease or with a *T. solium* extract. Splenocytes were removed from mice carrying cysticercosis infection for 6 weeks as well as from normal, non-infected mice of the same age. The normal cells were exposed to Conconavalin-A (Con-A). The cells from the infected mice were exposed to the purified *T. solium* cysteine protease and the starting *T. solium* extract. Cellular proliferation was measured by 3H-thymidine incorporation. The purified protease elicited a proliferative response in the immune cells that was significantly greater than that elicited by the extract, equaling stimulation by the mitogen Conconavalin-A (Fig. 5).

Example IV.

Identification of Bound, Potent, and Selective Inhibitors Against the *T. crassiceps* and *T. solium* Cysteine Proteases *in Vitro*

It was shown in Example II that the cyst wall proteases from *T. solium* and *T. crassiceps* are biochemically similar. Thus, a protease inhibitor directed against the *T. solium* cyst wall cysteine protease would be expected to inhibit the cyst wall cysteine proteinase of *T. crassiceps*.

Our results with a Langmuir binding isotherm revealed that the *T. solium* cysteine proteinase has only one substrate binding site, suggesting that designing a specific inhibitor was feasible. Our goal in this study was to demonstrate efficacious, potent and selective inhibition of the enzyme *in vitro*, preferably characterized by binding of the inhibitor at the active site. Inhibitors that bind at the active site of an enzyme can also be used to assess the similarities of the enzymes' active sites (Fig. 6). Three inhibitors of *Taenia* cyst wall cysteine protease were identified, and the protective potential of two of these inhibitors *in vivo* in the treatment of murine cysticercosis was also evaluated.

Two protease inhibitors inhibited the purified *T. solium* cysteine proteases in an assay using Z-Phe-Arg-AFC as a substrate at low efficacious concentrations: Z-Leucine-Leucine-Leucine-Fluoromethylketone (Z-LLL-FMK) and Z-Leucine-Leucine-Tyrosine-Fluoromethylketone (Z-LLL-FMK). At nanomolar levels, these protease inhibitors suppressed the *T. solium* cysteine protease's activity by 100% and 97%, respectively. Our kinetic data show that these inhibitors were bound to the cysteine protease's active site and were also potent for its inactivation. A third inhibitor, Ph2-LF-KP was also noted to inhibit the target cysteine protease rather efficaciously, by 80% when used at the same concentration as Z-LLL-FMK and Z-LLY-FMK. The inhibitors (Z-LLL-FMK and Z-LLY-FMK) similarly inhibited the *T. crassiceps* cysteine protease.

Z-LLY-FMK and Z-LLL-FMK also inhibited degradation of human IgG by the *T. solium* cysteine protease. Thus, we hypothesized that they may also have the potential to block human IgG degradation by *Taenia* cysts *in vivo*. This consideration, the inhibitors' impressive efficacy in inhibiting the target proteases at low concentrations, and their nontoxicity to immune cells *in vitro* (Example VII) suggest that these inhibitors would serve as good candidates for chemotherapy in the mouse model of cysticercosis.

Example V.

Protection of BALB/c Mice from Cysticercosis Infection using the Specific Inhibitors Z-Leucine-Leucine-Tyrosine Fluoromethylketone (Z-LLY-FMK) and Z-Leucine-Leucine-Leucine-Fluoromethylketone (Z-LLL-FMK)

Based upon the premise that the cyst wall cysteine protease may serve a critical function to the life cycle of the *Taenia* cyst, we tested our most efficacious inhibitors, Z-LLL-FMK, Z-LLY-FMK and Ph2-LF-KP for their abilities to protect mice from cysticercosis infection. Z-LLY-FMK and Z-LLL-FMK were individually tested as prophylactics in a preliminary trial with BALB/c mice. Injections were carried out in 150 *ul* of dimethyl sulfoxide. Mice

were pre-injected intraperitoneally for two days with the inhibitor (~1.4 X 10-^{2}M, dissolved in an injection vol. of 150 ul 0.15M PBS) followed by infection with 10 *T. crassiceps* cysts/mouse in 200 *ul* 0.15 M PBS. Mice were subsequently dosed daily with the same concentration for four weeks. After one month of infection, both groups were euthanized and cysts removed by washing with sterile 0.15 M PBS. A minimum of two attached cysts were scored as multilobed (ML). Upon visual inspection, no mice showed effects of inhibitor toxicity.

Mice in groups treated with Z-LLL-FMK were protected 100% from cysticercosis infection (Table 3). Mice in groups treated with Z-LLY-FMK were protected 85%-97% from cysticercosis infection, in comparison to untreated controls (Tables 3 and 4). Mice in groups treated with Ph2-LF-KP were protected 40 %. A subsequent study repeated the identical prophylactic experiment with Z-LLY-FMK and demonstrated similar protection data (75%-90%).

Table 3. Correlation between *in vitro* inhibition of *T. solium* cysteine protease and *in vivo* protection

Inhibitor	*% Inhibition of T. solium CP in vitro*	*% Prophylactic protection of BALB/c mice*	*% Therapeutic protection of BALB/c mice*	*Immune cells observed by SEM on cyst surfaces*
Z-LLL-FMK	100%	100%	60%	N/a
Z-LLY-FMK	97%	85-97%	n/a	Intense
Ph2-LF-KP	80%	40%	n/a	None observed

"n/a" refers to unavailable data.

Table 4. The effect of Z-Leu-Leu-Tyr-Fluoromethylketone treatment of BALB/c mice challenged with *Taenia crassiceps* cysts

Untreated controls	Cyst #	Avg	SD	% Prot.	# ML	% ML	Avg
Mouse 1	240	197.7	34.3		30	12.5 %	14.4%
Mouse 2	156				16	10.3%	
Mouse 3	197				40	20.3%	
Treated controls							
Mouse 4	7	21.3	10.2	96.5%	4	57%	51%
Mouse 5	30			84.8%	13	43%	
Mouse 6	27			86.4%	14	52%	

Interestingly, a high percentage of cysts which did survive the treatment demonstrated abnormal morphology. Many of these cysts exhibited apoloar multilocularity (multi-lobed appearances), an abnormal budding pattern. Under histological exam, multilobed cysts showed enlarged walls compared to normal lobed cysts.

A therapeutic study with Z-LLL-FMK was also conducted. During treatment phase, each treated mouse received 1.48 X 10^{-2} *ug/ul* of Leu-Leu-Tyr-FMK daily for three weeks and every other day during the last week. The results revealed 60% protection when inhibitor treatment began two weeks after mice had been infected with *T. crassiceps* cysts (Table 3). Mice in all groups survived without noticeable side effects (e.g., coat condition, tail dragging or paresis, etc).

Finally, it is notable that a dose response of *in vitro* inhibition of the *T. solium* cysteine protease correlates with *in vivo* protection in BALB/c mice. Moreover, cysts removed from mice treated with the less effective inhibitor, Ph2-LF-KP, had neither adherent immune cells nor damaged tegument.

Example VI. Morphological Evaluation of Cysts in Mice Treated with Inhibitor of *Taenia* Cysteine Protease Activity

Scanning electron microscopy (SEM) examination of the few surviving cysts which were removed from mice treated with Z-LLY-FMK in Example V revealed the presence of adherent immune cells, which were concomitantly present where the cyst wall appears to be undergoing destruction (Fig. 7). We identified macrophages, neutrophils, fibroblasts, and collagenous deposits on the cyst surfaces from treated mice, in comparison to their absence on cysts from untreated mice. General surface atrophy was also apparent. Microtriches (microvilli) were either sloughed off in entire regions or significantly shortened in general, compared to cysts which were removed from untreated mice. Breaks in the integrity of the cyst surface were also noted for the cysts removed from treated mice. Higher magnification revealed tegumental erosion near immunological cells. Cysts removed from untreated mice were characterized by no immune cells, intact and longer microtriches, and homogeneity of the cyst surface. These results are consistent with other studies which have shown that viable cysticerci in pig muscle and those removed from humans at autopsy show little surrounding host inflammation.

Example VII.

Z-LLY-FMK and Z-LLL-FMK are Nontoxic to Splenocytes from BALB/c Mouse Cells *in Vitro*

Mouse splenocytes were removed and treated with either inhibitor alone or inhibitor and conconavalin A (Con-A). Splenocytes from normal mice were removed and treated with either inhibitor alone, or inhibitor and conconavalin A. Proliferation was measured by ^{3}H-thymidine incorporation into the cells. A complete dose response curve for the inhibitor was conducted, with the highest dose representing in Fig. 8. The inhibitors did not induce a proliferative response (as measuared by ^{3}H-thymidine incorporation). Furthermore, the inhibitors did not affect the responsiveness of the splenocytes to Con-A.

Moreover, this dosage is three times the concentration which was utilized to inject mice on a daily basis in the prophylactic experiments (Example V).

Furthermore, trypan blue staining demonstrated that the mouse cells were all viable, even if the inhibitor was present. These studies further support our observation that no host side effects were observed in mice when they were treated with the specific inhibitors, *in vivo*. Z-LLL-FMK and Z-LLY-FMK thus appear to be nontoxic to normal BALB/c mouse splenocytes *in vitro*.

Example VIII.

Delivery of *Taenia* Cysteine Protease Inhibitor to Humans

Inhibitors for medical or veterinary use are designed to inhibit the activity of *Taenia* cysteine protease in humans, in pigs or in both. In pigs, *T. solium* cysts are located predominantly in muscles. However, the parasite is very neurotropic in humans, hence the disease often manifests itself as neurocysticercosis. In neurocystercercosis the *T. solium* cysts are located in the brain, thus it is desirable to design inhibitors that are able to cross the blood-brain barrier. The murine *T. crassiceps* model is well-suited for determining the *in vivo* susceptibility of tapeworm larvae (cysts) to drugs, but potential drugs for human use also need to be evaluated for their ability to cross the blood-brain barrier. Drugs like albendazole and praziquantel that affect brain cysts are surmised to cross the barrier.

It has been experimentally shown that the oil-water partition coefficient of nonelectrolytes is correlated with their membrane permeability and brain uptake in animal models (Levin, J. Med. Chem., 23:682-684(1980); Cornford, Exper. Parasitol., 70:25-34 (1990)). Therefore, partition coefficients for the inhibitor compounds will be determined, and if their values predict barrier impermeabilty, strategies are pursued to improve the likelihood of permeation. A variety of strategies for by-passing this barrier are known in the art, involving chemical modification of drugs, physical alteration of the blood/brain barrier and circumvention of the blood-brain barrier (reviewed in Abbott et al., Mol. Med.

Today, March 1996, 106-113). For example, Alkermes, Inc. of Cambridge, MA, a company that specializes in drug-delivery systems, has a promising blood-brain barrier permeabilizing drug, RMP-7 (Cereport®), that is now in Phase II of clinical trials. As another example, the candidate drugs can be are chemically modified to improve their partition coefficients. The FMK substituent, for example, is thought to be important in membrane permeability of the drug and may assist in transporting the drug across the blood-brain barrier.

The complete disclosures of all patents, patent applications including provisional patent applications, and publications, and electronically available material (e.g., GenBank amino acid and nucleotide sequence submissions) cited herein are incorporated by reference. The foregoing detailed description and examples have been provided for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described; many variations will be apparent to one skilled in the art and are intended to be included within the invention defined by the claims.